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In this study, neuroprotective ganglioside derivatives are examined so they can be targeted to specific points in cell death pathways. Here, ganglioside functional groups required for neuroprotection and blood-brain barrier permeance are determined. Cell death mechanisms and the mechanism(s) by which semisynthetic gangliosides intervene in the cell death process are also studied. In the second year C2, C4, C8, C14, C20, and C26 fatty acid GM1 derivatives were synthesized. Experiments on semisynthetic ganglioside cytoprotection using a retinoic acid-differentiated SH-SY5Y human neuroblastoma cell/MPP<sup>+</sup> model have been initiated. Each derivative requires analysis at different concentrations and preincubation times. Of the compounds studied to date (C2GM1, dichloroC2GM1, C4GM1, C14GM1), the C4GM1 derivative is the most cytoprotective, being 1.4 times more effective than the parent GM1. cDNA microarray analysis of changes in gene expression associated with MPP<sup>+</sup> toxicity revealed that MPP<sup>+</sup> exposure resulted in decreased RMB3 expression, increased GADD153 expression, and increased c-Myc expression. Further findings suggest that increased pancreatic protein disulfide isomerase (PDIp) expression in MPP<sup>+</sup> stressed SH-SY5Y cells may promote Lewy body formation and perhaps contribute to neurotoxicity and neurodegeneration.

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## INTRODUCTION

Natural and semisynthetic gangliosides may protect neurons prior to, and following, neurotoxin exposure. The hydrophilic property of gangliosides, however, restricts their blood-brain barrier (BBB) permeability when given peripherally. This hinders their use as neuroprotective agents. Gangliosides are amenable to chemical derivatization so that semisynthetic derivatives with both cytoprotective properties and improved ability to cross the BBB can be produced. For example, gangliosides with C2, or dichloro-C2, short chain fatty acids in the ceramide moiety are more cytoprotective than the parent ganglioside GM1; and the electrochemically neutral internal ester of GM1 crosses an endothelial cell model of the BBB significantly better than its parent compound (1). This study examines ganglioside functional group derivatives that provide cytoprotection AND effectively cross the BBB; information that will provide a basis for future studies of neuroprotective mechanisms. This research studies the ability of ganglioside derivatives to be cytoprotective in *in vitro* models using the dopaminergic neurotoxin, 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>) and the SH-SY5Y human neuroblastoma cell line. Derivatives determined to have therapeutic potential are tested *in vitro* for their ability to cross a brain capillary endothelial cell culture model of the BBB. Derivatives that are both cytoprotective and that effectively cross the *in vitro* BBB model will be tested *in vivo* for their ability to neuroprotect dopaminergic neurons in both chronic and acute neurotoxicity models using the MPP<sup>+</sup> precursor, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). This research studies the hypothesis that "changes in ganglioside ceramide and/or oligosaccharide functional groups will improve neuroprotection through changes in cytoprotection and BBB transcytosis." This research will provide a basis to improve ganglioside neuroprotection in neurodegenerative diseases, e.g., Parkinson's disease, and neurotoxin exposure.

## BODY

### Response to reviewer's comments on first annual report

The P.I. appreciates the thoughtful review of the first annual report received on July 24, 2003. The following are responses to the three major issues addressed by the reviewer (in *italics* below).

- (1) *It would be important to have kinetic data on the redistribution of GM1 intracellular trafficking from plasma membrane to mitochondria during MPP<sup>+</sup> toxicity.* Indeed, it is extremely important to understand the intracellular redistribution of GM1 and its derivatives. In this regard, we have established a collaboration with Dr. Wayne Lencer (see letter appended) to examine these issues. Dr. Lencer has obtained interesting data that implies the ceramide acyl group of gangliosides plays a seminal role in the determination of intracellular targets for ganglioside redistribution. It may also

be, however, that gangliosides manifest their cytoprotective effects at sites other than mitochondria. They may, for example, modulate intracellular calcium levels, alter growth factor synthesis, or other cellular functions that could render stressed cells more viable. For this reason, additional measures of cell viability (other than the MTT assay for mitochondrial reductive capacity) are being instituted. Because gangliosides and their derivatives alter plasma membrane physicochemical properties, dye-based or enzyme-based assays of cell status that involve plasma membrane integrity do not, alone, adequately reflect the viability of the cell population. Therefore, several assays need to be included in the determination of the most effective neuroprotective ganglioside derivatives. In addition to the MTT assay and neurochemical analysis of catecholamines as originally proposed, we will also include other measures of cell status such as tyrosine hydroxylase, DAPI staining, internal calcium concentrations, dopamine transporter, caspase-3, or intact nuclei counts.

- (2) *Although the publications included are interesting in that they indicate genes that may be involved in cytotoxicity, they are only indirectly related to the thrust of the proposed neuroprotection studies and are not relevant to any of the specific hypotheses.* The first part of this project requires a significant time investment to the synthesis of the ganglioside derivatives. Publications based on the use of the derivatives must, of course, await their synthesis. In vitro experiments are now underway and it is anticipated that publications should be forthcoming. During this labor-intensive portion of the project, the P.I. has contributed to the studies on differential gene expression in the MPP<sup>+</sup> model. These studies are related to the Statement of Work for this project because they will help to delineate the mechanisms of ganglioside derivative action and to guide the in vivo studies proposed for the latter part of the project.
- (3) *Although there is no discrepancy with the original grant proposal, progress has been quite slow in the proposed semisynthetic synthesis of ganglioside derivatives, and it is not clear that the biological part, the test system to be used, is capable of realizing significant protective differences. Review or even a visit by a qualified chemist/cell biologist team might strengthen the possibility that the specific aims will be accomplished.* Progress in the semisynthesis of ganglioside derivatives has progressed at a steady rate. The recent purchase of a chemical synthesizer should help expedite future syntheses as reaction conditions can be better controlled. To address the issue of the test system, we have established a collaboration with Dr. Richard Fine, a highly respected cell biologist from Boston University and the Bedford VA Hospital (letter of collaboration appended). In addition, it is anticipated that other dopaminergic cell culture systems will be examined as possible test systems for the derivatives, e.g., MN9D cells.

This project consists of 4 specific objectives in the **Statement of Work**.

**Statement of Work, Objective 1- Semisynthetic ganglioside derivatives will be synthesized from gangliosides isolated and characterized in the P.I.'s laboratory. These will include derivatives of the ceramide fatty acids, oligosaccharide functional groups, including internal esters, asialo, and reduced carboxylic acid (gangliosidol), and combinations of ceramide and oligosaccharide derivatives. Syntheses will be performed as described in Methods. Semisynthetic ganglioside derivatives will be characterized by chemical and mass spectrometric techniques.**

The yields of ganglioside derivatives have been improved by the purchase of a chemical synthesizer that allows better control of reaction conditions including the ability to maintain an inert gaseous atmosphere for the synthesis of lysoGM1, the precursor to the ceramide fatty acid derivatives. In the second year of this proposal, several ganglioside derivatives have been synthesized. Additional quantities of the C2, dichloroC2, C4, C8, C14, and C20 derivatives have been produced. The C26 derivative will be synthesized soon. In addition, oligosaccharide derivatives of GM1 have been made including asialoGM1 and the GM1 internal ester. Syntheses of oligosaccharide derivatives of the semisynthetic gangliosides are in progress.

**Statement of Work, Object 2 - Semisynthetic derivatives that specifically retain or improve the cytoprotective properties of the parent compound will be determined by testing them *in vitro* using an MPP<sup>+</sup> model of neurotoxicity in SH-SY5Y cells.**

The experimental paradigm to test cytoprotective compounds has been modified to accommodate the different preincubation time and concentration requirements of the various derivatives. Now, incubations are performed at three different ganglioside derivative preincubation times and at three different derivative concentrations.

Cytoprotection of ganglioside GM1 and its derivatives are determined by plating 10,000 SH-SY5Y cells in each well of three 48-well plates with DMEM media and 10% fetal calf serum. Retinoic acid is included in the media to induce differentiation. Differentiated cells are used because they more closely represent a nondividing neuronal phenotype. After 6 days in culture the medium is changed to DMEM with 0.5% serum and GM1 or the derivatives are added at to plates designated as 0 min, 30 min. or 120 min. preincubation. Low serum content is utilized to avoid excessive ganglioside binding to serum proteins. The lower serum content does not alter cell viability as determined by the MTT assay. Using this paradigm, several derivatives have been tested. These include the C2 (LIGA4), dichloroC2 (LIGA20), C4, and C14 fatty acid derivatives and asialoGM1. Of these, the C4 derivative with 2 hr

preincubation has shown the most efficacious cytoprotection compared to the parent GM1. The C4 derivative provided cytoprotection that was 1.4 times greater than GM1 (Figure 1).

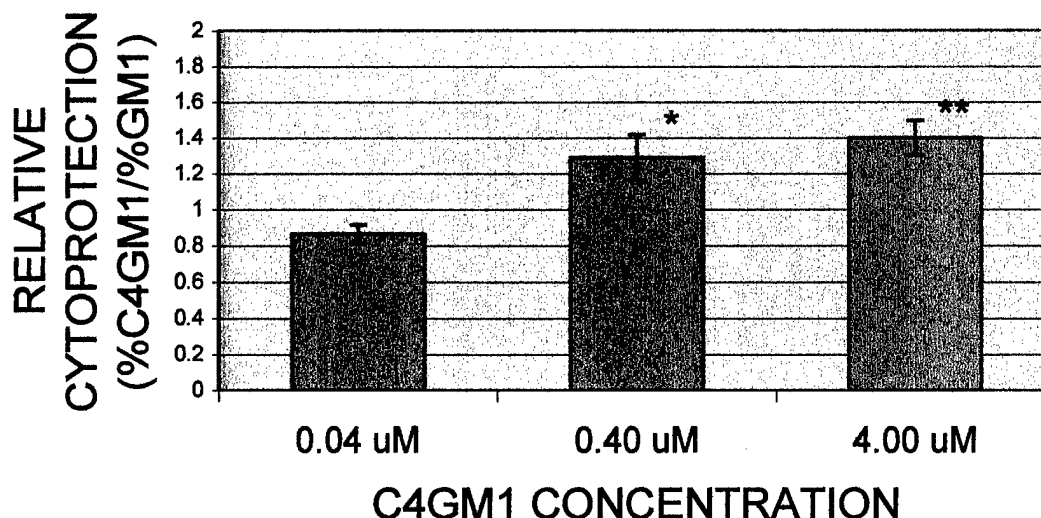


Figure 1. Relative cytoprotection by C4GM1. Control cells (no MPP<sup>+</sup>, no glycolipid) or cells exposed to 1mM MPP<sup>+</sup> were incubated with either GM1 or C4GM1. Cell viability was estimated using the MTT assay. Relative Values are the percent control of MPP<sup>+</sup>-exposed cells with C4GM1 divided by the percent control of MPP<sup>+</sup> cells exposed to GM1.

\* Differs from GM1 protected cells, One-way ANOVA, Tukey-Kramer post-hoc test,  $p < 0.05$ ,  $N = 5$

\*\* Differs from GM1 protected cells, One-way ANOVA, Tukey-Kramer post-hoc test,  $p < 0.01$ ,  $N = 4$

In the last Annual Report it was noted that lysoGM1 retained the cytoprotective properties of GM1 and that this may indicate that the ceramide acyl chain does not contribute significantly to cytoprotection or that the mechanism of cytoprotection may differ between GM1 and lysoGM1. The current C4GM1 data indicate that, indeed, the ceramide fatty acyl chain does play a significant role in cytoprotection and that future studies on cytoprotective mechanisms must account for the possibility that different, and possibly additive, mechanisms of cytoprotection may be utilized by the different derivatives. In addition, the ceramide fatty acid will influence blood-brain barrier penetrance that will be determined in future studies. In addition to the MTT assay and neurochemical analysis of catecholamines as originally proposed, additional assays to estimate cell viability are now being instituted. These will include some or all measures of tyrosine hydroxylase, DAPI staining, internal calcium concentrations, dopamine transporter, caspase-3, and intact nuclei.

Additional experiments have been performed (in collaboration with Dr. Kelly Conn and others at the VA Hospital, Bedford, MA) to further establish the mechanism of MPP<sup>+</sup> toxicity, which will help in the delineation of mechanisms by which ganglioside derivatives are cytoprotective. Previous work in this area has shown that mitochondrial dysfunction (2) and endoplasmic reticulum (ER) stress (3) result from MPP<sup>+</sup> exposure in SH-SY5Y cells. To further define MPP<sup>+</sup> toxic mechanisms and Parkinson's disease neuronal degeneration, cDNA microarray analysis was used to characterize the transcriptional response of retinoic acid-differentiated SH-SY5Y cells to MPP<sup>+</sup> exposure. Retinoic acid-differentiated SH-SY5Y cells decreased RNA binding protein 3 (RMB3) expression, increased GADD153 expression, and increased the proto-oncogene c-Myc expression when exposed to MPP<sup>+</sup>. These genes may be important factors in MPP<sup>+</sup> toxicity through mechanisms involving translation (4), gene splicing (5), or transcriptional activation.

Several ER stress genes were also examined in retinoic-acid differentiated SH-SY5Y cells following MPP<sup>+</sup> exposure. RT-PCR and Western blot analysis showed that the protein disulfide isomerase (PDI) family member PDIp, previously thought to be expressed exclusively in the pancreas, was upregulated within twelve hours of MPP<sup>+</sup> exposure. Immunohistochemical studies on postmortem brain tissue identified the presence of PDIp in Lewy bodies of Parkinson's disease and dementia with Lewy bodies, co-localizing with alpha-synuclein. Thus, PDIp expression in stressed neurons may contribute to the toxic mechanism of MPP<sup>+</sup> and to neurodegeneration in Parkinson's disease.

**Statement of Work, Objective 3, Effective cytoprotective semisynthetic ganglioside derivatives that effectively cross a brain capillary endothelial cell model of the blood-brain barrier (BBB) will be determined. Model BBB transcytosis will be assessed by liquid scintillation counting of radiolabeled derivatives in aliquots taken from the lower wells of Transwell cell culture plates.** These studies were initiated in the second half of year 2. Initial studies, however, have been slowed due to the discontinued commercial availability of the transwell inserts used in previous studies (1). Modifications of the culture system had to be made so that tight junctions could maintain their integrity during the course of the experiments. Tests with FITC molecular markers and ohmmeter readings over time have shown that tight junctions are maintained for several hours and the system is now ready to be used for the determination of GM1 derivative BBB permeance.

**Statement of Work, Objective 4, Semisynthetic ganglioside derivatives that effectively protect neurons *in vivo* using chronic and acute MPTP administration models of neurotoxin insult will be determined by testing, in mice, those derivatives that both cytoprotect the SH-SY5Y cells from MPP<sup>+</sup> toxicity *in vitro* AND that effectively cross the *in vitro* BBB model.** The chronic and acute models



represent apoptotic and necrotic cell death mechanisms, respectively. Neuroprotection will be evaluated by neurochemical analysis of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA), and by neuronal counts of Nissl substance, tyrosine hydroxylase (TH) and dopamine transporter (DAT) positive neurons of the substantia nigra pars compacta. Work on this objective is now scheduled to begin in the second half of year three.

## **KEY RESEARCH ACCOMPLISHMENTS**

1. Additional syntheses of fatty acid derivatives
2. Synthesis of oligosaccharide derivatives including the internal ester and asialo compounds
3. Further optimization of incubation conditions for screening derivative cytoprotection in retinoic acid differentiated SH-SY5Y cells
4. Tested several fatty acid derivatives with the C4GM1 (N-butyl) derivative showing the greatest cytoprotection to this time.
5. Optimized conditions for the endothelial cell blood-brain barrier model using new culture inserts because the old inserts are no longer commercially available.
6. cDNA microarray analysis of changes in gene expression associated with MPP<sup>+</sup> toxicity revealed decreased RMB3 expression, increased GADD153 expression, and increased c-Myc expression.
7. Increased pancreatic protein disulfide isomerase (PDIp) expression in MPP<sup>+</sup> stressed SH-SY5Y cells

## **REPORTABLE OUTCOMES**

### Manuscripts

1. Conn,K, Doherty S, Eisenhauer P, Fine R, Wells J, Ullman MD: Neuroprotective Ganglioside Derivatives, *Annals of the New York Academy of Sciences*, 991: 330-332 (2003).
2. Conn,K., Ullman MD, Larned MJ, Eisenhauer PB, Fine RE, Wells JM: cDNA Microarray Analysis of Changes in Gene Expression Associated with MPP<sup>+</sup> Toxicity in SH-SY5Y Cells, *Neurochem. Res.* In press.

## Abstracts

3. Conn K.J., Gao W., McKee A., Lan M.D., **Ullman M.D.**, Eisenhauer P.B., Johnson R.J., Fine R.E., Wells J.M.: Identification of the protein disulfide isomerase family member PDip in experimental and idiopathic Parkinson's disease. Neuroscience Meetings, New Orleans, LA (2003)

## CONCLUSIONS

Our experience in synthesizing and developing HPLC procedures to purify semisynthetic ganglioside derivatives provides the opportunity to find improved cytoprotective ganglioside derivatives, to define required functional groups for cytoprotection, and to initiate studies to determine cytoprotective mechanisms. Preliminary findings indicated that GM1, lysoGM1, and LIGA20 had essentially the same cytoprotective capacity that implied the fatty acid moiety may be of minimal importance in the cytoprotective mechanism. Additional studies on fatty acid derivatives, however, have shown that the C4 (N-butyl) derivative has greater cytoprotective properties than any of the previously tested derivatives as determined by using the MTT assay. As described, additional measures of cytoprotection in this model system are being instituted and examination of the fatty acid derivatives and their oligosaccharide modifications will continue into the third year. Initial studies on the endothelial cell model of the blood-brain barrier were slowed because the commercial availability of the transwell inserts used in previous studies (1) were discontinued and modifications of the culture system had to be made.

Based on previous results, endoplasmic reticulum stress may play an important part in MPP<sup>+</sup> toxicity (3). Further investigation into the differential expression of ER stress genes in retinoic acid-differentiated SH-SY5Y cells by Western blot analysis showed that the protein disulfide isomerase (PDI) family member PDip, previously identified as exclusively expressed in pancreatic tissue, is upregulated within 12 hours following MPP<sup>+</sup> exposure. Immunohistochemical studies on post mortem brain tissue identified the presence of PDip in Lewy bodies of Parkinson's disease and dementia with Lewy bodies, co-localizing with alpha-synuclein. These findings suggest that increased PDip expression in stressed dopaminergic neurons may promote Lewy body formation and perhaps contribute to neurodegeneration. This emphasizes the importance of understanding the mechanisms of MPP<sup>+</sup> toxicity in attempts to further define mechanisms of ganglioside neuroprotection because by better understanding neurotoxic and neuroprotective mechanisms, specific steps in the cell death process can be targeted.

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3. Conn KJ, Wen-Wu G, Ullman MD, et al. Upregulation of GADD153/CHOP in MPP+-treated SH-SY5Y cells. *J.Neurosci.Res.* 2002;68:755-760.
4. Derry JM, Kerns JA, Francke U. RBM3, a novel human gene in Xp11.23 with a putative RNA-binding domain. *Hum.Mol.Genet.* 1995;4:2307-2311.
5. Hofmann Y, Wirth B. hnRNP-G promotes exon 7 inclusion of survival motor neuron (SMN) via direct interaction with Htra2-beta1. *Hum.Mol.Genet.* 2002;11:2037-2049.

## **APPENDICES**

### **1. Letter of Collaboration**

Dr. Wayne Lencer, Children's Hospital, Boston, MA

### **2. Letter of Collaboration**

Dr. Richard Fine, Boston University, Boston, MA

### **3. Journal article**

Conn K, Doherty S, Eisenhauer P, Fine R, Wells J, Ullman MD: Neuroprotective Ganglioside Derivatives, *Annals of the New York Academy of Sciences*, 991: 330-332 (2003).

### **4. Journal Article**

Conn K., Ullman MD, Larned MJ, Eisenhauer PB, Fine RE, Wells JM: cDNA Microarray Analysis of Changes in Gene Expression Associated with MPP<sup>+</sup> Toxicity in SH-SY5Y Cells, *Neurochem. Res.* In press.

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September 3, 2003

M. David Ullman, Ph. D.

VA Hospital (182B)

200 Springs Road

Bedford, MA 01730

Dear David,

This letter is to confirm my interest and enthusiasm to collaborate with you in studies on the structure and function of gangliosides in eukaryotic cell biology. As you know from our previous meetings, my laboratory is extremely interested in the kinetics of ganglioside redistribution during intracellular trafficking from the plasma membrane to Golgi and ER. Our belief is that the mechanism and intracellular targets of ganglioside redistribution are dependent on the ceramide acyl composition. The collaboration between our laboratories should provide extremely important insights into this and several other aspects of ganglioside cell biology, including the mechanisms of ganglioside cytoprotection. This is extremely exciting work. I look forward enthusiastically to our continued collaboration.

Best regards,

Wayne Lencer, MD



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9/3/2003

Dear David:

Your Parkinson's disease grant is extremely interesting and important and I will be more than happy to consult with you on those relevant cell biology issues. Gangliosides have long been known to be neuroprotective and your approach should go a long way in defining compounds that traverse the blood-brain barrier and that are neuroprotective in the MPTP model. The work will also establish a strong foundation for understanding the mechanism of ganglioside neuroprotection and I am looking forward to continuing our long-standing professional relationship. Our collaborations, over the years, have been very productive and I have every reason to believe that this project will be highly rewarding as well.

Sincerely,

A handwritten signature in cursive script that reads "Richard E. Fine".

Dr. Richard E. Fine  
Professor of Biochemistry and  
Neurology

## Neuroprotective Ganglioside Derivatives

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<sup>c</sup>University of Massachusetts Medical School Shriver Center, Waltham,  
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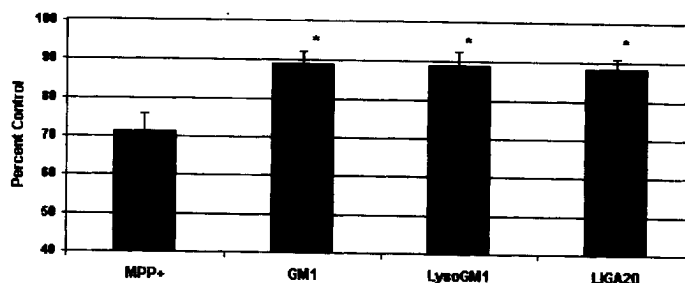
**KEYWORDS:** gangliosides; neuroprotective agents

Natural and semisynthetic gangliosides protect neurons from injury. The hydrophilic property of gangliosides, however, restricts their blood-brain barrier (BBB) permeability when given peripherally. This hinders their use as neuroprotective agents. Gangliosides are amenable to chemical derivatization, so that semisynthetic derivatives with both cytoprotective properties and improved ability to cross the BBB can be produced. Therefore, ganglioside functional group derivatives that provide cytoprotection and effectively cross the BBB are being sought. This will provide a basis to understand neuroprotective mechanisms. Insight into neuroprotective mechanisms also requires an understanding of cell death processes. Thus, studies into 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>)-induced changes in gene expression are also ongoing.

Semisynthetic GM1 derivatives were synthesized and tested for their ability to protect SH-SY5Y human neuroblastoma cells from MPP<sup>+</sup> toxicity. SH-SY5Y cells were cultured at 37°C in a 95% air, 5% CO<sub>2</sub> humidified incubator and maintained in DMEM-high glucose supplemented with 10% fetal bovine serum. For cytoprotection experiments,  $10 \times 10^3$  cells per well were plated in a 48-well cell culture plate and differentiated in the presence of 10  $\mu$ M retinoic acid. After 4 days, medium was replaced with that containing 0.5% fetal calf serum and the test ganglioside (690 nM) 1 hour before exposure to MPP<sup>+</sup>. Ganglioside was again added on the day after MPP<sup>+</sup> treatment.

For experiments on gene expression,  $5 \times 10^5$  cells were plated in 100 mm<sup>2</sup> culture dishes in 10 mL of DMEM medium containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 mg/mL streptomycin and cultured for 4 days. Freshly prepared toxin was added to the cultures and incubated for the requisite times. RNA iso-

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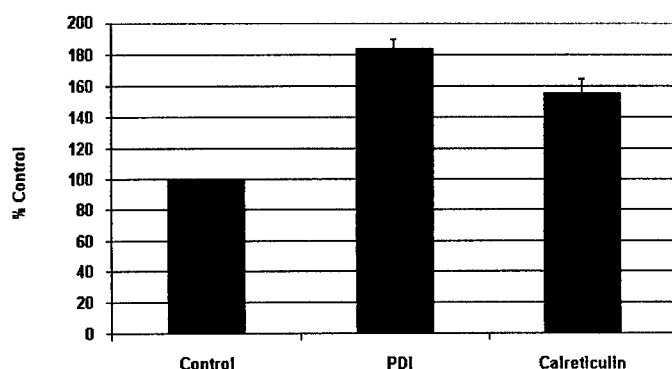
**FIGURE 1.** GM1, LysoGM1, and LIGA20 cytoprotection. Under the conditions described in the text, GM1, lysoGM1, and LIGA20 showed comparable cytoprotection. Values are percent control  $\pm$  SEM.  $n = 4$ . \*Differs from MPP<sup>+</sup> only, one-way ANOVA, Tukey-Kramer post-hoc test,  $P < 0.05$ .

lation and RT-PCR microarray and Western blot analyses have previously been described.<sup>1,2</sup>

For cytoprotection experiments, dose response and preincubation experiments performed with GM1 indicated that a 1-hour preincubation of 690 nM GM1 provided maximal cytoprotection against 1 mM MPP<sup>+</sup>. This same preincubation period and concentration was used for lysoGM1 (GM1 amine, no ceramide fatty acid) and LIGA20 (GM1 with a dichloroacetate ceramide fatty acid). GM1, lysoGM1, and LIGA 20 were tested for their ability to protect SH-SY5Y cells from MPP<sup>+</sup> toxicity, and they showed comparable cytoprotection (Fig. 1). Further testing, however, is required to examine the effects of preincubation time and concentration on ganglioside-derivative cytoprotection. Nonetheless, these results imply that the ceramide fatty acid does not significantly influence cytoprotection in this model system or that the derivatives have different mechanisms of action. The ceramide fatty acid, however, most likely contributes significantly to blood-brain barrier permeability.

To better understand possible ganglioside-derivative cytoprotective mechanisms in cell death processes, the toxic effects of MPP<sup>+</sup> on mitochondrial gene expression were initially investigated in undifferentiated SH-SY5Y cells. It was found that MPP<sup>+</sup> decreased expression of NADH:ubiquinone oxidoreductase (complex I) subunit 4 (ND4), a mitochondrial gene important for electron transport chain complex I function.<sup>1</sup> MPP<sup>+</sup> did not affect expression of other mitochondrial (16S and COX1) or nuclear (B14) genes, indicating a degree of specificity for MPP<sup>+</sup>-induced decreased ND4 expression.

Gene microarray analysis (Clontech) also indicated that MPP<sup>+</sup> exposure increased the endoplasmic reticulum (ER) stress-related gene GADD153. RT-PCR analysis demonstrated that GADD153 mRNA levels increased linearly up to 72 hours. Western blot analysis indicated that GADD153 protein levels increased to 24 hours, and caspase 3 activation increased linearly from 24–72 hours.<sup>2</sup> This suggests that increased GADD153 expression and ER stress may also contribute to the initiation of an active cell death mechanism in the SH-SY5Y cells. In parallel cultures treated with toxins whose primary mode of action is either via mitochondrial impairment (rotenone) or via oxidative stress (6-hydroxy dopamine or H<sub>2</sub>O<sub>2</sub>),



**FIGURE 2.** Expression of select ER stress genes in SH-SY5Y cells after exposure to MPP<sup>+</sup> (1 mM). RT-PCR analyses were performed using RNA isolated 72 hours after exposure to 1 mM MPP<sup>+</sup>. Gene expression was normalized to G3PDH. Values are percent control  $\pm$  SEM.

GADD153 expression was not increased.<sup>2</sup> This supports the possibility that a cellular mechanism different from mitochondrial impairment or oxidative stress—for example, ER stress—contributes to MPP<sup>+</sup> toxicity. In further support of an ER-stress-related mechanism, MPP<sup>+</sup> increases the expression of at least two other ER stress genes, protein disulfide isomerase (PDI) and calreticulin (FIG. 2). Thus, MPP<sup>+</sup>-induced cell death may entail multiple pathways, and perhaps successful neuroprotection will require multiple therapeutic agents and/or therapeutic agents with multiple mechanisms of action such as gangliosides.<sup>3</sup> Knowledge of functional group requirements for ganglioside cytoprotection and a better understanding of cell death mechanisms will provide a basis for delineating their cytoprotective mechanisms and for targeting molecular structures to specific components of cell death processes.

#### ACKNOWLEDGMENTS

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⊛ Add grant info

## cDNA Microarray Analysis of Changes in Gene Expression Associated with MPP<sup>+</sup> Toxicity in SH-SY5Y Cells

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cDNA microarray analysis of 1-methyl-4-phenyl-pyridinium (MPP<sup>+</sup>) toxicity (1 mM, 72 h) in undifferentiated SH-SY5Y cells identified 48 genes that displayed a signal intensity greater than the mean of all differentially expressed genes and a two-fold or greater difference in normalized expression. RT-PCR analysis of a subset of genes showed that c-Myc and RNA-binding protein 3 (RMB3) expression decreased by ~50% after 72 h of exposure to MPP<sup>+</sup> (1 mM) but did not change after 72 h of exposure to 6-hydroxydopamine (25  $\mu$ M), rotenone (50 nM), and hydrogen peroxide (600  $\mu$ M). Exposure of retinoic acid (RA)-differentiated SH-SY5Y cells to MPP<sup>+</sup> (1 mM, 72 h) also resulted in a decrease in RMB3 expression and an increase in GADD153 expression. In contrast, c-Myc expression was slightly increased in RA-differentiated cells. Collectively, these data provide new insights into the molecular mechanisms of MPP<sup>+</sup> toxicity and show that MPP<sup>+</sup> can elicit distinct patterns of gene expression in undifferentiated and RA-differentiated SH-SY5Y cells.

**KEY WORDS:** cDNA microarray; Parkinson's disease; 1-methyl-4-phenyl-pyridinium; gene expression; neurodegeneration.

### INTRODUCTION

Parkinson's disease (PD) is a slowly progressing neurodegenerative disorder with no clear etiology. Pathological hallmarks of the disease include the death of dopaminergic neurons of the substantia nigra (SN) and

the presence of Lewy bodies ( $\alpha$ -synuclein and ubiquitin-positive, eosinophilic, cytoplasmic inclusions) in the surviving neurons. Recent etiological study in twins (1) strongly suggests that environmental factors play an important role in typical, nonfamilial PD, beginning after age 50. Environmental factors reported to increase the risk of developing PD include rural living, well water use, and exposure to pesticides, herbicides, industrial chemicals, and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (2). Environmental factors alone are not sufficient to cause PD. It has been hypothesized that PD is the result of environmental factors acting on genetically susceptible individuals against a background of aging. Three genetic risk factors have been associated with PD. These include polymorphisms in the ubiquitin C-terminal hydrolase gene (3), deletions in the parkin gene (4), and mutations in the  $\alpha$ -synuclein gene (5,6). Mutations, deletions, and polymorphisms in these genes, however,

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account for a very small number of PD cases. The majority of idiopathic, sporadic cases of PD are likely caused by a complex pattern of environmental and genetic contributions from unidentified genes.

*1-human*  
*out*  
The toxins MPTP and  $MPP^+$  have been used to model PD neurodegeneration experimentally in nonhuman primates and mice. The neurotoxic MPTP metabolite,  $MPP^+$ , is actively transported into dopaminergic neurons by dopamine transporters (7), where it is concentrated in the mitochondria (8). There it inhibits complex I of the electron transport chain (ETC) (9). The resultant impairment of ATP generation results in dysregulated calcium homeostasis (10), mitochondrial membrane depolarization (10), free radical production (11), and ultrastructural changes to the endoplasmic reticulum (ER) (12–14). MPTP and  $MPP^+$  are neurotoxic to cells in culture. For example, exposure of undifferentiated human SH-SY5Y neuroblastoma cells for 72 h to 1 mM  $MPP^+$  induces mitochondrial dysfunction (15), reactive oxygen species (ROS), and apoptotic death (16).

Previous work in our laboratory suggested that mitochondrial dysfunction (17) and ER stress (18) accompanying  $MPP^+$ -toxicity in undifferentiated human SH-SY5Y neuroblastoma cells may be controlled, in part, by changes in gene expression. In an effort to better understand the mechanisms of cell death during  $MPP^+$  toxicity and PD neurodegeneration, we have used cDNA microarray analysis to characterize the transcriptional response of undifferentiated SH-SY5Y neuroblastoma cells to  $MPP^+$ . We identified transcription factors and cell cycle proteins as major classes of proteins differentially expressed in undifferentiated SH-SY5Y cells exposed to  $MPP^+$ . In addition, we identified two genes, c-Myc and RNA binding protein 3 (RMB3), whose differential expression has not previously been associated with  $MPP^+$  toxicity in SH-SY5Y cells. Exposure of retinoic acid (RA)-differentiated SH-SY5Y cells to  $MPP^+$  (1 mM, 72 h) also resulted in a decrease in RMB3 expression and an increase in GADD153 expression. In contrast, c-Myc expression was slightly increased in RA-differentiated cells. These data provide new insight into the molecular mechanisms of  $MPP^+$  toxicity and show that  $MPP^+$  can elicit distinct patterns of gene expression in undifferentiated and RA-differentiated cells.

## EXPERIMENTAL PROCEDURES

**Materials.**  $MPP^+$  iodide, 6-OHDA, rotenone, Dulbecco's modified Eagle's medium (DMEM), retinoic acid (RA) (Sigma-Aldrich, St. Louis, MO, USA),  $H_2O_2$  (Fisher Scientific, Rochester, NY, USA),

and fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, USA) were purchased from commercial sources.

**Cell Culture, Differentiation, and Toxin Treatment.** The undifferentiated human neuroblastoma cell line SH-SY5Y (ATCC CRL-2266) was cultured at 37°C in a 95% air, 5%  $CO_2$  humidified incubator and maintained in DMEM (high glucose) supplemented with 10% FBS. Undifferentiated cells were routinely subcultured when confluent, and the culture medium was changed twice a week. For toxin experiments in undifferentiated cells,  $0.5 \times 10^6$  cells were plated into 100 mm<sup>2</sup> dishes (Corning, Cambridge, MA, USA) in 10 ml DMEM plus 10% FBS, 100 units/ml penicillin, and 100 mg/ml streptomycin and cultured for 4 days. Freshly prepared toxins were added to the cultures and incubated at 37°C for various lengths of time. For toxin experiments with cells differentiated with RA,  $0.8 \times 10^6$  cells were plated into 100 mm<sup>2</sup> dishes (Corning, Cambridge, MA, USA) in differentiation media (10 ml DMEM plus 10% FBS, 100 units/ml penicillin, 100 mg/ml streptomycin, and 10  $\mu M$  RA). Cells were cultured for 3 days; then the medium was changed to 10 ml fresh differentiation medium. The cells were cultured another 7 days, and freshly prepared  $MPP^+$  or vehicle, phosphate buffered saline (PBS), was added for various lengths of time in fresh differentiation medium. All appropriate safety precautions were used in handling toxin solutions.

**RNA Isolation and Reverse Transcription.** Total RNA was isolated using Tri Reagent (Sigma). The integrity of each RNA preparation was monitored by ultraviolet visualization of ethidium bromide-stained RNA following electrophoresis on 1% agarose-formaldehyde gels. Approximately 50  $\mu g$  of RNA was treated with 1 unit of DNase-I using the MessageClean kit (GenHunter Corp, Nashville, TN, USA). DNase-treated RNA was subjected to PCR analysis, as described below, using primers to glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12; G3PDH) to ensure that each RNA preparation was free of DNA. DNase-treated RNA (5  $\mu g$ ) was reverse transcribed using oligo(dT) primers provided in the Superscript kit (Clontech, Palo Alto, CA, USA).

**Microarray Analysis.** The conditions for microarray analysis have been previously described (18). Briefly, cells were collected after 72 h from vehicle (control) and  $MPP^+$ -treated undifferentiated SH-SY5Y cells. Total RNA was isolated, and radioactive  $^{32}P$  was incorporated into cDNA in a reverse transcription reaction using gene-specific primer sequences (Clontech). Radiolabeled cDNA was hybridized with human toxicology 1.2 array membranes (Clontech), and differential gene expression was visualized by exposure to phosphorimaging cassettes. Results were analyzed using Atlas Image 2.0 software (Clontech). The background value was determined by the default external method in which the background was set at the median intensity of the "blank space" between the different panels of the array. The signal threshold was set by the background-based signal threshold method in which the adjusted intensity (intensity of the spot minus background) was at least 1.67  $\times$  the background value. The normalization coefficient was determined using the global sum method, which adds the values of signal over background for all genes on the array.

**PCR Amplification, Visualization, and Quantification.** Primers for the amplification of the G3PDH, GADD153, Histone H4, c-Myc, and RMB3 cDNA were purchased (Clontech or Gibco BRL, Grand Island, NY, USA). Conditions for G3PDH and GADD153 amplification were identical to those previously described (18). For the Histone H4, c-Myc, and RMB3 primer sets, each reaction cycle consisted of the following steps: 94°C for 1 min, 60°C for 1 min and 72°C for 2 min. Histone H4, c-Myc, and RMB3 reactions were carried out for 30 cycles. Following resolution by electrophoresis on 2% agarose gels containing 0.5  $\mu g/ml$  ethidium bromide, PCR products were visualized and quantified using the 4400 ChemImager low-light imaging

system (Alpha-Innotech, San Leandro, CA, USA). c-Myc, GADD153, and RMB3 expression was expressed as a ratio to the value of G3PDH product obtained from parallel reactions.

**Data analysis.** Data from replicate experiments are presented as mean  $\pm$  SEM as specified.

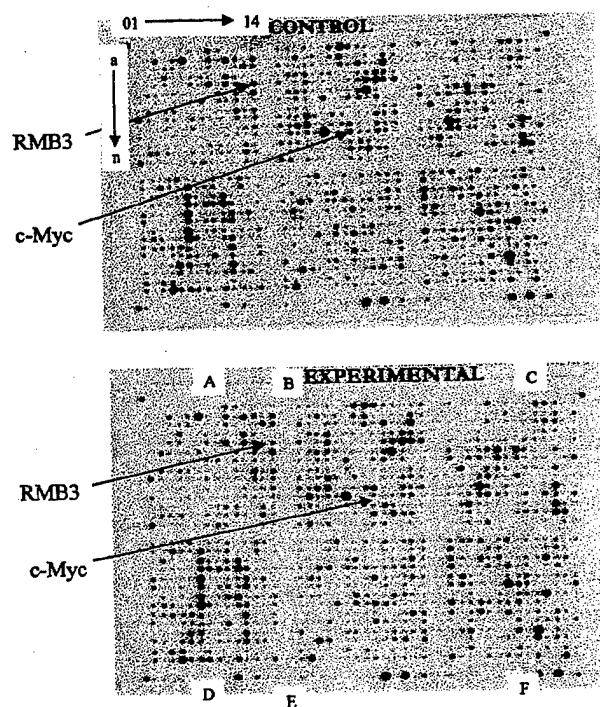
## RESULTS

cDNA microarray analysis was used to generate a gene expression profile of MPP<sup>+</sup> toxicity at 72 h after exposure (Fig. 1). In total, 1185 genes were screened in a single experiment for differential expression (positive or negative) induced by MPP<sup>+</sup>. Of these genes, 872 were determined to be either not expressed or unchanged using a signal threshold of 1.67. Of the 313 differentially expressed genes, 48 met the cut-off criteria recommended by the manufacturer for RT-PCR confirmation: a signal intensity greater than the mean of all differentially expressed genes and a two-fold or greater differ-

ence in normalized expression using a normalization coefficient of 1.53. Of these 48 genes, 16 were chosen for RT-PCR confirmation based on the magnitude of differential expression identified in the array experiment. These genes, their gene bank identification, their membrane coordinates, their fold difference in expression (up or down) as determined by the gene microarray experiment, and their difference in expression (expressed as % control) as determined by RT-PCR are shown (Table 1). The RT-PCR data represented in Table 1 are the combination of data collected from four independent experiments and represents at least triplicate RT-PCR reactions.

Division of these 48 genes based on the primary classification code from the array manufacturer showed that approximately half of all the differentially expressed genes could be divided into one of three classifications: transcription proteins (14.6%), cell cycle proteins (14.6%), and kinase activators/inhibitors (14.6%). Proteins associated with transcription (basic transcription factors, activators, and repressors) comprised 33.3% of all the downregulated genes and 8.3% of all the upregulated genes and included YY1, twist-related protein, PGK1, RMB3, U2 small nuclear ribonucleoprotein, c-Jun proto-oncogene, and c-Myc proto-oncogene. Proteins associated with the cell cycle (cyclin dependent kinase inhibitors, cell cycle regulating kinases, other cell cycle proteins) represented 8.3% of all the downregulated genes and 16.7% of all the upregulated genes and include CLK3, p21, NEF2-related factor 1, asparagine synthetase, inhibitor of DNA binding protein 3, inhibitor of DNA binding 1 protein, and PTMA. Lastly, 14.6% of the 48 were kinase activators/inhibitors. These genes represented none of the downregulated genes and 19.4% of all the upregulated genes and include 14-3-3 protein, Ems1 oncogene, PKC11, STAT-induced inhibitor 2, CRYM, phospholipase C  $\gamma$ -binding protein, and tuberlin.

Other classes of genes identified include proteins associated with apoptosis (GADD45, glutathione S-transferase theta 2, GADD153, HSIH2, and structure-specific recognition protein 1), growth factors, cytokines, hormones, or interleukins (interleukin 2 receptor alpha subunit, DLK, GPI, vascular endothelial growth factor and ribonuclease/angiogenesis inhibitor), intracellular kinases, G proteins, or phosphatases (mitogen-activated protein kinase 5, protein kinase C alpha polypeptide, PP2A and Ras-related C3 botulinum toxin), metabolism (COMT, transglutaminase, glutamic oxaloacetic transaminase 1, and bifunctional methylenetetrahydrofolate), heat shock/chaperones proteins (heat shock 70-kD protein 1, mitochondrial stress-70 protein, and T-complex protein 1 epsilon), and oncogenes/tumor suppressors (SIP and EAR3). Other gene classes included cell surface antigens, extracellular



**Fig. 1.** Phosphorimages from control and MPP<sup>+</sup> array membranes. Shown are phosphorimages of gene microarray membranes hybridized with radiolabeled cDNA generated from RNA isolated 72 h after exposure of undifferentiated SH-SY5Y cells to vehicle (control) and MPP<sup>+</sup>. Panels A to F are shown on the experimental membrane. The coordinates a-n and 01-14 are shown on panel A of the control membrane. Two examples of differential gene expression (c-Myc and RMB3) are shown by the arrows at the coordinates B09k and A14e, respectively.

Table I. Differential gene expression induced by 1 mM MPP<sup>+</sup> in undifferentiated SH-SY5Y cells at 72 h.

Coord.	Up	Down	Name	Gene bank	RT-PCR
A02f	2.6		Galactosidase-binding lectin 3	M35368	ND
A08n	2.7		Yin & yang (YY1)	X70683	ND
A09a		2.8	Twist-related protein	X99268	84.8 ± 0.671
A14b		2.7	Phosphoglyceride kinase 1 (PGK1)	V00572	94.5 ± 1.88
A14e		7.8	<b>RNA binding protein 3 (RMB3)</b>	<b>U28686</b>	<b>44.2 ± 1.50</b>
A14j	2.0		U2 small nuclear ribonucleoprotein B	M15841	ND
B01j	2.0		CDC-like kinase 3 (CLK3)	L29220	ND
B02k	6.1		CDK inhibitor (p21)	U09579	161 ± 7.11
B02n	2.2		NEF2-related factor 1	U08853	ND
B04d	4.7		Asparagine synthetase	M27396	127 ± 3.23
B04e	2.6		Inhibitor of DNA-binding protein 3	X69111	ND
B04f	3.0		Inhibitor of DNA binding 1 protein	D13889	ND
B04i		2.0	Prothymosin alpha (PTMA)	M26708	93.6 ± 1.30
B08j	5.8		IGF binding protein 5	M65062	148 ± 3.19
B09j	5.3		Jun proto-oncogene	J04111	181 ± 27.3
<b>B09k</b>		<b>13.5</b>	<b>Myc proto-oncogene</b>	<b>V00568</b>	<b>49.5 ± 1.97</b>
B12g		2.4	SYT-interacting protein (SIP)	AF080561	ND
B12k		2.3	v-erbA related protein 3 (EAR3)	X12795	ND
B14a		2.3	Heat shock 70-kD protein 1	M11717	ND
B14j	5.1		Mitochondrial stress-70 protein	E15189	132.7 ± 3.9
C06h	2.1		solute carrier family 12 member 4	U55054	ND
C14g	2.3		Membrane-bound and soluble COMT	M65212	ND
D01j	2.4		Transglutininase	M98252	ND
D01n	2.6		Glutamic oxaloacetic transaminase 1	M37400	ND
D02c	6.4		Bifunctional methylenetetrahydrofolate	X16396	125 ± 5.95
D04m	2.4		T-complex protein 1 epsilon	D43950	ND
D05i	2.1		Ribophorin II	Y00282	ND
D07d	4.5		GADD45	M60974	190 ± 13.4
D10f		2.0	Glutathione S-transferase theta 2	L38503	ND
<b>D10g</b>	<b>29</b>		<b>GADD153</b>	<b>S40706</b>	<b>332 ± 12.7</b>
D10h	2.4		HSIAH2	U76248	ND
D11d	2.0		Structure-specific recognition protein 1	M86737	ND
D13l		9.0	Interleukin 2 receptor alpha subunit	X01057	ND
E02e		4.7	Delta-like protein (DLK)	U15979	ND
E03d		2.5	Glucose-6-phosphate isomerase (GPI)	K03515	96.1 ± 2.22
E04d	4.8		Vascular endothelial growth factor	M32977	147 ± 4.71
E04l	2.0		Ribonuclease/angiogenesis inhibitor	M36717	ND
E08m	2.2		Mitogen-activated protein-kinase 5	U25265	ND
E10h	2.7		Protein kinase C alpha polypeptide	M22199	ND
E12g	7.8		Protein phosphatase (PP2A)	M64930	ND
E13i	2.0		Ras-related C3 botulinum toxin	M29870	ND
E14l	2.4		14-3-3 protein	X57346	ND
F01a	2.1		Ems1 oncogene	M98343	ND
F01b	2.0		Protein kinase C inhibitor 1 (PKCII)	U51004	ND
F01f	3.3		STAT-induced STAT inhibitor 2	AB004903	ND
F01n	2.0		Mu-crystallin homolog (CRYM)	L02950	ND
F02a	2.4		Phospholipase C gamma-binding protein	AB005216	ND
F02e	2.0		Tuberin	X75621	ND

Note: For each gene the gene bank identification, membrane coordinate (Coord.), fold difference in differential expression (up or down), and the difference in expression (expressed as % control) as determined by RT-PCR are shown. ND denotes those genes for which RT-PCR was not determined. Of the genes tested by RT-PCR, three showed differential expression in excess of two-fold: c-Myc, GADD153, and RMB3 (Bold type).

transport carrier proteins, gradient-driven transporters, and protein modification enzymes represented by the genes for galactosidase-binding lectin 3, IGF binding protein 5, solute carrier family 12 member 4, and ribophorin II, respectively.

Of the 16 genes tested by RT-PCR, three showed differential expression in excess of two-fold: GADD153, c-Myc, and RMB3. Previous characterization of GADD153

expression in undifferentiated SH-SY5Y cells demonstrated that GADD153 expression increases after 24 h of MPP<sup>+</sup> exposure and precedes activation of caspase 3 (18). To determine the time course of c-Myc and RMB3 differential expression, undifferentiated SH-SY5Y cells were treated with and without 1 mM MPP<sup>+</sup> for 24, 48, 72, and 96 h. Cells at each time point were isolated for RT-PCR analysis. Equal concentrations of RNA from

each experimental condition were reverse transcribed into cDNA and amplified by PCR. Figure 2 is representative of two independent experiments. The spot densitometry value of quadruplicate PCR products was expressed as percentage of no MPP<sup>+</sup> control  $\pm$  SEM. After 1 mM MPP<sup>+</sup> exposure, c-Myc and RMB3 steady-state mRNA were decreased after 24 h ( $92.3 \pm 1.89$ ,  $81.0 \pm 1.65$ , respectively), 48 h ( $82.4 \pm 0.767$ ,  $83.02 \pm 1.13$ ,

respectively), 72 h ( $53.7 \pm 1.76$ ,  $44.2 \pm 1.53$ , respectively) and 96 h ( $8.85 \pm 4.75$ ,  $23.4 \pm 5.89$ , respectively).

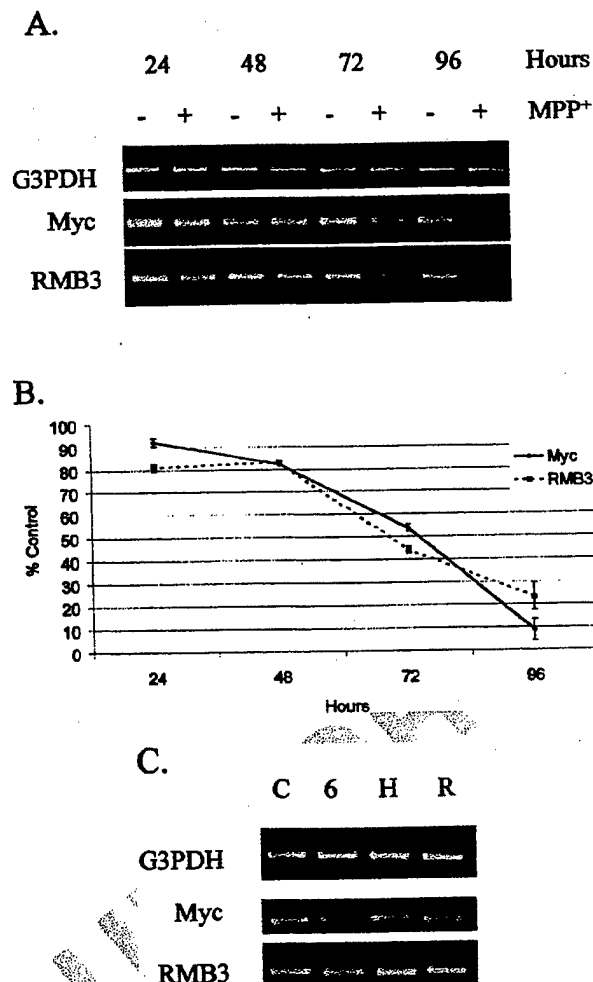
The specificity of the c-Myc and RMB3 response was examined in undifferentiated SH-SY5Y cells treated with other toxins. RT-PCR analyses performed using RNA isolated from cells treated 72 h with the LD<sub>50</sub> concentrations (18) for 6-OHDA (25  $\mu$ M), H<sub>2</sub>O<sub>2</sub> (600  $\mu$ M), or rotenone (50 nM) showed no differences in c-Myc and RMB expression (Fig. 2C). RT-PCR analysis using RNA isolated at 5, 24, 48, and 96 h after exposure to 6-OHDA, H<sub>2</sub>O<sub>2</sub>, or rotenone also did not show any changes in c-Myc and RMB3 expression, compared to RNA isolated from control cells (data not shown).

Because both GADD153 (19) and Myc (20) are proteins that have been implicated in the control of the cell cycle, we determined whether MPP<sup>+</sup> altered the expression of these genes in RA-differentiated cells. SH-SY5Y cells were exposed to 10  $\mu$ M RA (21) and on days 1–9 after exposure, cells were photographed (Fig. 3A) and RNA was isolated for RT-PCR analyses using primers to G3PDH and histone H4 (Fig. 3B). Histone H4, but not G3PDH, gene expression increased from days 1 to 4, and this increased gene expression corresponded to an increase in cell density. After 5 days of RA exposure, histone H4 gene expression decreased and remained constant through day 9 (Fig. 3B). The cell density also remained constant from days 5 through 9 (Fig. 3A). Thus 6 days of RA exposure was sufficient to induce a differentiated (nondividing) phenotype.

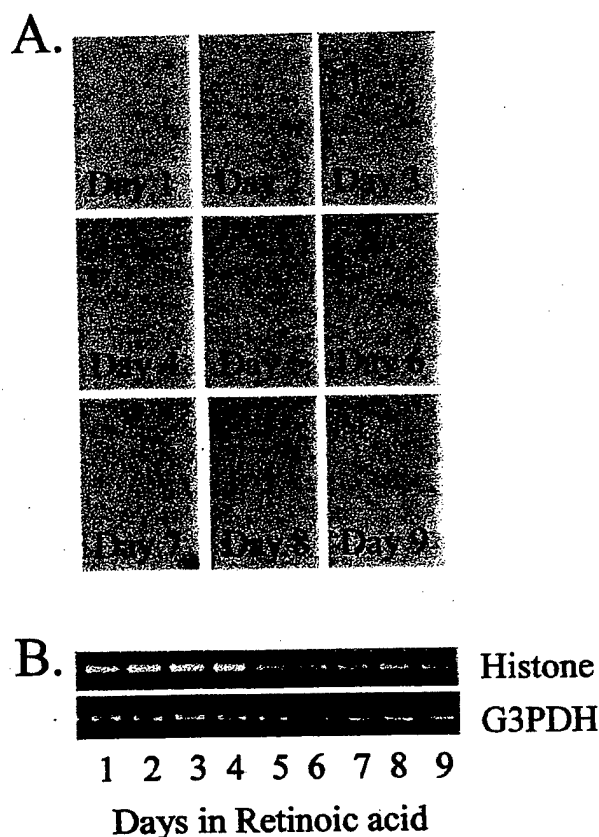
Using these differentiation conditions, we isolated RNA from differentiated cells treated with MPP<sup>+</sup> for 72 h and measured gene expression of GADD153, c-Myc, and RMB3 by RT-PCR. Compared to control (% control  $\pm$  SEM) GADD153 ( $151.1 \pm 5.80$ ) and RMB3 ( $44.6 \pm 5.29$ ) showed changes in gene expression similar to those of undifferentiated cells (Fig. 4). In contrast, c-Myc showed a slight increase compared to control ( $119.2 \pm 2.79$ ).

## DISCUSSION

cDNA microarray analysis was used to identify genes whose differential expression may contribute to the molecular mechanisms of MPP<sup>+</sup> toxicity and PD neurodegeneration. When undifferentiated SH-SY5Y cells were challenged for 72 h with MPP<sup>+</sup>, ~4% of the genes tested showed differential gene expression. This percentage is similar to other cDNA microarray studies using similar experimental design and cut-off criteria for differential



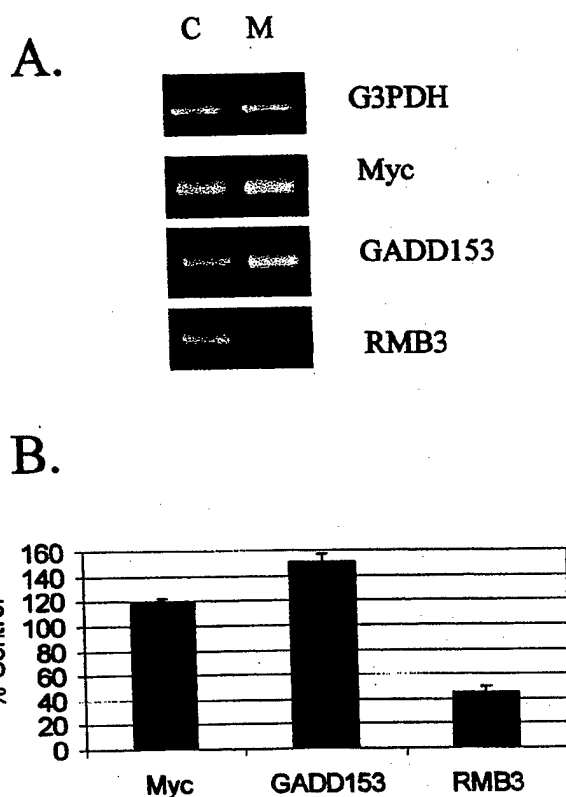
**Fig. 2.** Time course and specificity of c-Myc and RMB3 expression in undifferentiated SH-SY5Y. **A.** Electrophoretic separation of PCR products from a typical RT-PCR experiment is shown using primers to G3PDH, RMB3, and c-Myc and RNA isolated 24, 48, 72, and 96 h after exposure to 1 mM MPP<sup>+</sup>. **B.** Quantification of c-Myc and RMB3 steady-state mRNA was performed as described in the text. Data are graphed as percent control  $\pm$  SEM, where control represents those cells not treated with MPP<sup>+</sup>. Values represent the mean of quadruplicate PCR products. **C.** Electrophoretic separation of PCR products from a typical RT-PCR experiment is shown using primers to G3PDH, RMB3, and c-Myc with RNA isolated 72 h after exposure to the LD<sub>50</sub> concentrations of 6-OHDA (6), H<sub>2</sub>O<sub>2</sub> (H), and rotenone (R).



**Fig. 3.** Time course of cell division induced by differentiation of SH-SY5Y cells with retinoic acid. **A.** Photographs of SH-SY5Y cells on days 1 through 9 following exposure to RA (10  $\mu$ M). **B.** Electrophoretic separation of PCR products from a typical RT-PCR experiment is shown using primers to G3PDH and histone H4 with RNA isolated on days 1 through 9 after RA exposure.

expression (22). RT-PCR confirmation of differential gene expression of a subset of genes showed that 15 of the 16 genes tested showed changes in gene expression (up or down) consistent with the array results; however, fold differences in gene expression were less robust (~25% of array values). This discrepancy in magnitude of differential expression between cDNA microarray and RT-PCR may reflect differences in hybridization conditions for each gene-specific primer set and the corresponding cDNA target. For example, in the array experiment a common hybridization condition is used for all of the genes tested, and in RT-PCR, hybridization conditions are optimized in a gene specific way (17).

Importantly, time course studies of differential gene expression of c-Myc and RMB3 show maximal changes in gene expression at time points other than 72 h. Therefore some of the genes identified as differentially



**Fig. 4.** Differential expression of GADD153, c-Myc, and RMB3 in RA-differentiated SH-SY5Y cells. **A.** Electrophoretic separation of PCR products from a typical RT-PCR experiment is shown using primers to G3PDH, c-Myc, GADD153, and RMB3 and RNA isolated 72 h after exposure to vehicle (C) or 1 mM MPP<sup>+</sup> (M). **B.** Quantification of c-Myc, GADD153, and RMB3 steady-state mRNA was performed as described in the text. Data are expressed as per cent control  $\pm$  SEM, where control represents those cells not treated with MPP<sup>+</sup>. Values represent the mean of quadruplicate PCR products derived from three independent experiments.

expressed at 72 h may show maximal changes in gene expression at earlier or later time points. These observations validate the microarray approach as effective in identifying genes whose differential expression accompanies MPP<sup>+</sup> toxicity, but also highlight the importance of confirmation and further analysis of changes in steady-state mRNA by another technique such as RT-PCR or Northern blot analysis.

In addition to identifying two genes (c-Myc and RMB3) whose differential expression has not previously been associated with MPP<sup>+</sup> toxicity in SH-SY5Y cells, we have used gene microarray analysis to identify transcription factors and cell cycle proteins as major classes of proteins differentially expressed by MPP<sup>+</sup> exposure. The fact that proteins involved in transcription were identified as differentially expressed in our system is consistent with

the notion that MPP<sup>+</sup> toxicity appears to be dependent on *de novo* protein synthesis (23,24). A number of different transcription factors have previously been identified as differentially expressed following MPP<sup>+</sup> exposure, including c-fos, fosB, Delta-fosB (25,26), and NF $\kappa$ B (27). Our observation that MPP<sup>+</sup> upregulates the expression of c-Jun is consistent with previous studies implicating the involvement of c-Jun in MPP<sup>+</sup>-mediated cell death. Prolonged c-Jun expression has been observed in both the striatum (26) and substantia nigra (28) following administration of MPTP in mice.

In this study we make the new observation that the transcription-related protein RMB3 (29) is downregulated following MPP<sup>+</sup> exposure. RMB3 encodes a polypeptide of predicted 17-kD molecular weight. Its putative RNA-binding domain most closely resembles that of two previously characterized human RNA-binding proteins, YRRM, the gene for which has been implicated in azoospermia, and hnRNP G, a glycoprotein, also identified as an autoantigen. hnRNP proteins are involved in controlling gene expression by binding RNA and influencing both translation and RNA splicing. For example, addition of hnRNP C1 in an *in vitro* translation system has been shown to enhance translation of c-Myc mRNA (30). If RMB3 was functioning in a similar manner in SH-SY5Y cells, reduced RMB3 expression could conceivably contribute to decreases in protein expression by inhibiting translation. Alternatively, RMB3 may function in a manner similar to hnRNP G by controlling RNA splicing (31). Differential mRNA splicing of the transcription factor XBP-1 has recently been shown to be a key regulatory step in controlling activation of the ER stress response, which has also been hypothesized to contribute to PD neurodegeneration (32).

In addition to RMB3, we also observed decreases in c-Myc gene expression. The c-Myc proto-oncogene plays a key role in proliferation, differentiation, apoptosis, and regulation of the cell cycle. c-Myc functions as a transcription factor positively or negatively regulating the expression of distinct sets of target genes. Transcriptional activation by c-Myc is mediated through dimerization with Max and binding to the DNA consensus sequence CA(C/T)GTG (the E-box). PC12 cells selected for resistance to MPTP show increased c-Myc expression (33), suggesting that c-Myc expression is important in ameliorating MPP<sup>+</sup>-toxicity. Although no modification in c-Myc expression has been observed in the surviving pigmented neurons of the SN in PD brains (34), this does not preclude the involvement of c-Myc in PD neurodegeneration. It is possible that surviving neurons do not show decreases in c-Myc expression and the neurons that downregulated c-Myc have died. The

inability to identify changes in c-Myc gene in some PD cases may be related to the fact that PD represents a chronic condition, with cell death occurring over a 10–20 year period at a rate of 5%–10% per decade. (35). In this case, changes in c-Myc expression may be rare at any given time point.

Decreases in c-Myc expression contribute to apoptosis (36,37). Decreased c-Myc expression could induce apoptosis by controlling the expression of other apoptosis-related genes. For example, c-Myc has been shown to downregulate GADD153 in rat fibroblasts (38) and to suppress induction of the GADD34, GADD45, and GADD153 by DNA-damaging agents (39). Alternatively, decreased c-Myc expression has been shown to induce apoptosis by effecting the expression of enzymes important in glutathione metabolism (40). Decreases in reduced glutathione have been implicated in both MPP<sup>+</sup> (41) and PD neurodegeneration (42).

Cell cycle proteins were another major class of polypeptides differentially expressed following MPP<sup>+</sup> exposure. For example, we observed increased expression of the cyclin-dependent kinase inhibitor p21. Increased p21 expression also has been observed in PC12 cells exposed to MPP<sup>+</sup> (43) and contributes, along with decreased c-Myc expression, to the inhibition of G1 to S phase progression of the cell cycle. Interestingly, we observed similar differential expression of RMB3 and GADD153 in both undifferentiated (dividing) and RA-differentiated (growth inhibited) cells. In contrast, c-Myc expression showed distinct patterns of differential expression in undifferentiated and RA-differentiated cells. One possibility is that MPP<sup>+</sup> exposure elicits unique cell death mechanisms in undifferentiated and RA-differentiated cells and that c-Myc participates in both pathways via unique downstream targets.

To gain insight into the type of cellular stress downregulating c-Myc and RMB3 expression after MPP<sup>+</sup> exposure, we compared c-Myc and RMB3 expression in parallel cultures treated with toxins whose primary mode of action is either via mitochondrial impairment (rotenone) or oxidative stress (6-OHDA or H<sub>2</sub>O<sub>2</sub>). The observation that none of these toxins decreased c-Myc and RMB3 expression suggests that a cellular mechanism unique from mitochondrial impairment or oxidative stress may contribute significantly to the downregulation of c-Myc and RMB3 by MPP<sup>+</sup>. Alternatively, the unique downregulation of c-Myc and RMB3 may reflect differences in the cell death pathway induced by each toxin. For example, MPP<sup>+</sup> has been shown to promote caspase 3 cleavage (16,18), whereas H<sub>2</sub>O<sub>2</sub> does not (44).

## CONCLUSION

In conclusion, the major findings of this study are: (i) transcription proteins and cell cycle proteins are major classes of polypeptides differentially expressed in undifferentiated SH-SY5Y cells after 72 h of MPP<sup>+</sup> exposure; (ii) in particular, c-Myc and RMB3 gene expression is specifically downregulated to ~50% in undifferentiated SH-SY5Y cells after 72 h of MPP<sup>+</sup> exposure; (iii) changes in c-Myc and RMB3 gene expression in undifferentiated cells are not caused by other neurotoxins such as 6-OHDA, H<sub>2</sub>O<sub>2</sub>, or rotenone, and; (iv) MPP<sup>+</sup> can elicit distinct patterns of gene expression in undifferentiated and RA-differentiated SH-SY5Y cells.

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